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## Estrous cycle fluctuations in sex and ingestive behavior are accentuated by exercise or cold ambient temperatures



Amir Abdulhay<sup>a,1</sup>, Noah A. Benton<sup>a,1</sup>, Candice M. Klingerman<sup>b</sup>, Kaila Krishnamoorthy<sup>c</sup>,  
Jeremy M. Brozek<sup>a</sup>, Jill E. Schneider<sup>a,\*</sup>

<sup>a</sup> Lehigh University, Department of Biological Sciences, Bethlehem, PA 18015, USA

<sup>b</sup> Bloomsburg University, Department of Biological and Allied Health Sciences, Bloomsburg, PA 17815, USA

<sup>c</sup> Drexel University Medical School, Philadelphia, PA 19104, USA

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### ABSTRACT

This article is part of a Special Issue “Energy Balance”.

In female Syrian hamsters (*Mesocricetus auratus*), low circulating levels of ovarian steroids are associated with increased food hoarding and decreased sexual motivation, but these effects are exaggerated in food-restricted females. To determine whether cold ambient temperature has the same effects as food restriction, groups of hamsters were fed ad libitum while they were housed at either 5 °C or 22 °C, and then tested for behavior for 90 min on each day of the estrous cycle. In females housed at 22 °C, high levels of sexual motivation and low levels of food hoarding were seen every day of the estrous cycle. In females housed at 5 °C, high levels of sexual motivation were restricted to the periovulatory day. On the three nonestrous days, these females showed high levels of food hoarding, but not food intake. A separate cohort of females were provided with access to running wheels and housed at 22 °C. They showed high levels of sexual motivation restricted to the periovulatory day, similar to the pattern of sexual motivation seen in cold-housed females. Unlike cold-housed females, those with running wheels showed low levels of food hoarding and high levels of food intake. Food restriction, cold housing, and access to wheels had no significant effect on plasma estradiol or progesterone concentrations, but significantly decreased plasma leptin concentrations. All three energetic challenges unmask estrous cycle fluctuations in sexual motivation that are obscured in laboratory conditions, i.e., isolation in a small cage with an overabundance of food.

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### Introduction

Levels of sex and ingestive behavior fluctuate over the ovulatory cycle, due in part to fluctuations in circulating ovarian steroid concentrations. In many different species, including Syrian hamsters (*Mesocricetus auratus*), high circulating concentrations of estradiol are associated with decreases in food intake and increases in sexual motivation (for sex behavior, reviewed by [Blaustein and Erskine, 2002](#); [Schneider et al., 2013](#); [Wallen and Tannenbaum, 1997](#); for food intake, reviewed by [Asarian and Geary, 2006, 2013](#); [Schneider et al., 2013](#); for the choice between food and sex, reviewed by [Schneider et al., 2013](#)). This is true for ovariectomized females treated with estradiol and for females in the preovulatory phase of the estrous/menstrual

cycle, the phase when circulating concentrations of estradiol are highest. These results are consistent with the idea that ovarian steroids modulate the behavioral sequence so that sex behaviors coincide with fertility and ingestive behaviors foreshadow mating, pregnancy, and lactation.

Ovarian steroid modulation of behavior might best be understood in the context of natural fluctuations in energy supply and demand. In natural environments, where food supplies and the energetic demands for thermogenesis and foraging vary annually or unpredictably, survival requires that individuals anticipate future energetic challenges by foraging, “overeating,” accumulating adipose tissue, and/or caching food in the home or burrow (reviewed by [Bronson, 1989](#)). In such environments, individuals risk starvation by choosing reproductive behaviors over foraging. Evolutionary adaptation, however, involves differential reproductive success as well as mere survival. Thus, during the fertile periovulatory period, females should be predisposed to mating, even in the face of moderate energetic challenges that promote foraging, eating, and hoarding at other times. Only when the energetic challenge is

\* Corresponding author at: Department of Biological Sciences, 111 Research Drive, Bethlehem, PA 18015, USA. Fax: +1 610 758 4004.

E-mail address: [js0v@lehigh.edu](mailto:js0v@lehigh.edu) (J.E. Schneider).

<sup>1</sup> Co-first authors.

severe, such as after periods of total food deprivation in lean animals, will the hypothalamic–pituitary–gonadal (HPG) system be fully inhibited (reviewed by Schneider, 2004; Wade et al., 1996). This suggests that in environments with mild to moderate energetic challenges, increases in circulating estradiol overcome the risks involved in reproduction by making sex behavior a priority over foraging, hoarding, and eating (reviewed by Schneider et al., 2013).

This perspective leads to some specific predictions that we have been testing using female Syrian hamsters as a model system. We predicted that the effects of ovarian steroids on various aspects of reproductive and ingestive behavior would be exaggerated when females were exposed to energetic challenges. Female Syrian hamsters are particularly appropriate for testing this idea because they display quantifiable sex and ingestive behaviors that fluctuate with remarkable regularity over their four-day estrous cycle (Ciaccio and Lisk, 1971; Huck et al., 1985; Johnston, 1977; Orsini, 1961). Furthermore, in nature, they live solitarily in widely-distributed burrows, compete vigorously for a limited number of these burrows, and travel long distances to forage for food and find mating partners (Gattermann et al., 2008). These activities are compressed into a short time period (less than 2 h) spent above ground each day, consistent with the idea that decisions about when to forage and hoard food and when to engage in mate searching and other reproductive behaviors are critical for survival and reproductive success (Gattermann et al., 2008). Ovarian hormones might play an important role in setting behavioral priorities from night-to-night over the estrous cycle. We thus designed an apparatus that simulates important aspects of the natural habitat and allows us to measure the female's preference for food-related or sex-related stimuli.

Using the preference apparatus, we tested the hypothesis that the effects of ovarian steroids on behavior are accentuated in energetically challenging environments (Klingerman et al., 2010). Specifically, females were either fed ad libitum or limited to 75% of their daily ad libitum food intake, a moderate energetic challenge that does not inhibit estrous cycles, lordosis, uterine weight, or ovarian steroid secretion (Klingerman et al., 2010). Consistent with our hypothesis, food-limited but not ad libitum-fed hamsters restrict their approaches to males and their appetitive sex behaviors to the periovulatory period, and spend the rest of the estrous cycle engaged in intensive food hoarding. By contrast, females fed ad libitum spend most of their time making vaginal scent marks near males throughout the estrous cycle and spend very little time hoarding food. Furthermore, the effects of food restriction are limited to appetitive behaviors (food hoarding and male preference); the same level and duration of food restriction has no significant effect on consummatory behaviors (food intake and lordosis) (Klingerman et al., 2010). These results are consistent with the idea that ovarian hormones prioritize behavioral motivation in energetically labile environments.

The present study tested the hypothesis that estrous cycle fluctuations in appetitive behaviors are amplified by other environmental manipulations. We measured sex and ingestive behavior over the estrous cycle in female hamsters living at cold ambient temperatures (5 °C). This manipulation was chosen because it increases thermogenic capacity, increases overall daily energy expenditure, and decreases body weight and adiposity, even in ad libitum-fed hamsters (Jefimow et al., 2004; Ruf and Graf, 2010; Schneider and Wade, 1990; Zhao, 2011). If our hypothesis were correct, changes in behavior over the estrous cycle would be amplified in hamsters housed at 5 °C, and masked in hamsters housed at 22 °C. In a separate cohort of females, we repeated the cold or warm exposure and determined whether there were significant differences in circulating concentrations of ovarian hormones, estradiol and progesterone, or the adipocyte hormone, leptin.

Next, we determined whether estrous cycle fluctuations in behavior would be amplified by another environmental manipulation, voluntary wheel running. This manipulation induces locomotion throughout the dark phase of the photoperiod, increases overall energy expenditure, increases food intake, and decreases body weight and adiposity (Coutinho

et al., 2006; Davis et al., 1987; Refinetti and Menaker, 1997; Richards, 1966). Furthermore, in a different hamster species (*Phodopus sungorus*), voluntary wheel running attenuates many aspects of reproduction by producing deficits in energy availability (Petri et al., 2010). We measured appetitive sex and ingestive behavior over the estrous cycle in female hamsters housed either with or without access to running wheels. If our hypothesis were correct, changes in behavior over the estrous cycle would be amplified in hamsters housed with access to running wheels, and flattened in hamsters housed without access to wheels. In a separate cohort of females, we repeated these voluntary exercise conditions and determined whether there were significant differences in circulating concentrations of estradiol, progesterone, and leptin.

## Methods

### *Methods common to all experiments*

#### *Animals and housing*

Experiments were conducted according to the guiding principles for research published by the National Institutes of Health, the Lehigh University Institutional Animal Care and Use Committee, and enforced by the United States Department of Agriculture. Female Syrian hamsters (*M. auratus*) between 109.3 and 180.4 g in body weight (approximately 90–210 days of age) were obtained from a colony bred at the Lehigh University animal facility (original generations of animals were obtained from Charles River Breeding Laboratories; Wilmington, MA) or purchased as adults from Charles River Breeding Laboratories. Animals were singly housed in opaque, Nalgene cages (31 × 19 × 18 cm) in a room maintained at 22 ± 1 °C with a 14:10 light–dark cycle (lights on at 2100 h).

#### *Hamster estrous cycles*

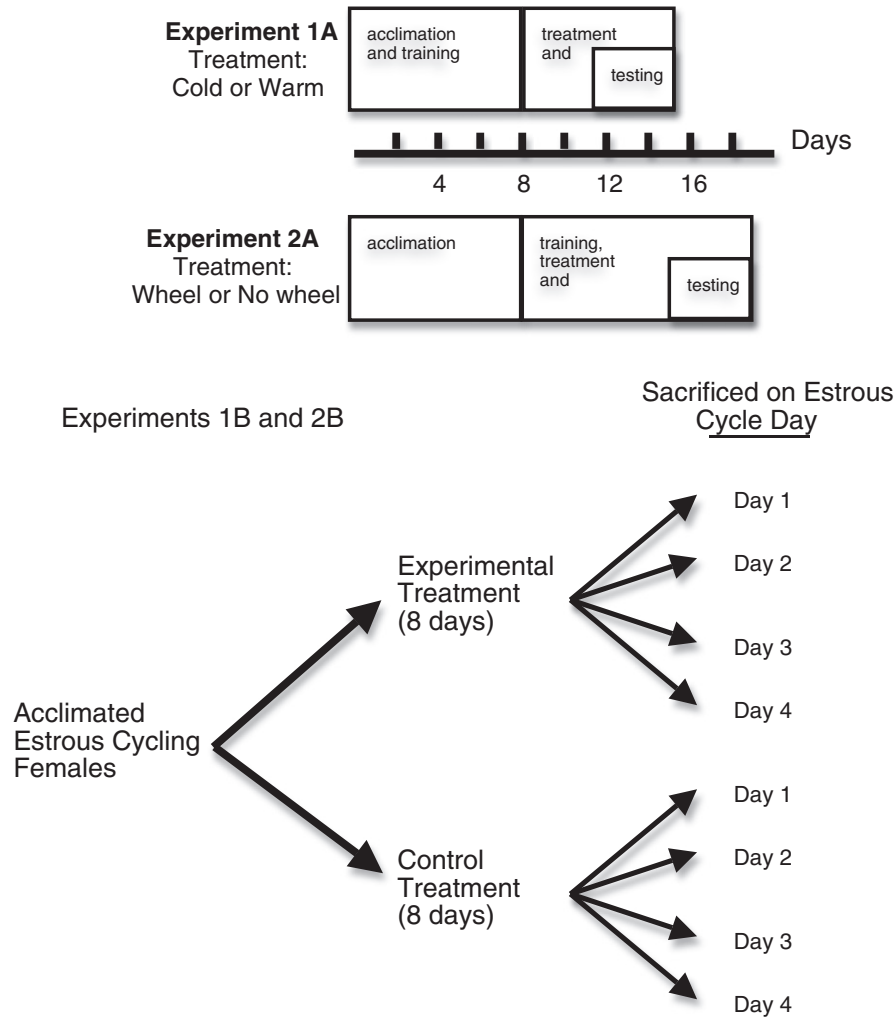
The Syrian hamster estrous cycle is four days long and ends with estrous behavior and ovulation the afternoon/evening of the fourth day. The day of estrous and ovulation is termed the “periovulatory day” in hamsters (which corresponds to “proestrous” in rats). In our experiments, estrous cycle day 1 is termed the “postovulatory day.” Day 2 is termed “follicular day 1.” Day 3 is termed “follicular day 2.” In Syrian hamsters, appetitive sex behaviors, such as vaginal scent marking, peak on follicular day 2, whereas lordosis occurs only on the periovulatory day (Takahashi and Lisk, 1983).

All hamsters showed at least two consecutive 4-day estrous cycles as determined by a positive test for sex behavior (the lordosis posture) with a sexually-experienced male hamster. The test for lordosis occurred within 1 h of the onset of the dark phase of the photo period each day.

#### *Experiment 1A: Effects of ambient temperature on behavior over the estrous cycle*

This experiment was designed to determine whether estrous cycle fluctuations in appetitive behaviors are amplified when animals are housed at low ambient temperatures known to increase thermogenic capacity and energy expenditure. Thus, estrous cycling hamsters were acclimated and trained at normal laboratory temperatures, housed in either the warm or cold for one week, and then tested for behaviors during their final 4 days in their respective environments (Fig. 1). The experiment was performed in 2 exact replicates approximately a year apart. Both replicates contained equal sample sizes of cold and warm-housed hamsters, except that the cold-housed group had 1 extra female in the second replicate.

The durations of cold exposure, ambient temperature, and feeding schedule were chosen to increase the energy required to maintain normal body temperature and to prevent the hamsters from becoming hypothermic. Syrian hamsters are known to become hypothermic when there is a confluence of factors: several weeks of cold exposure, short day lengths, and either food deprivation, or treatment with inhibitors



**Fig. 1.** A diagram of the chronology for experiments 1A and 2A (top) and for experiments 1B and 2B (bottom). In experiment 1A, females were acclimated to their home cages and then trained in the preference apparatus for 1.5–2 h each evening. Next, during the treatment period they were housed at either 5 or 22 °C. After 3 days of treatment, females were tested every day of the 4-day estrous cycle while they remained in their respective cold or warm room. In experiment 2A, females were acclimated then housed either with or without running wheels 24 h per day for the 10-day treatment period. They were trained in the preference apparatus for 1.5–2 h during the first part of the treatment period and tested on every day of the estrous cycle in the last part of the treatment period. In experiments 1B and 2B, females were acclimated and placed into treatment groups. In 1B, the groups were housed with or without running wheels. Treatments continued for 8 days and animals were euthanized and blood collected for analysis of plasma estradiol, progesterone, and leptin concentrations.

of fuel oxidation (Hoffman, 1968; Schneider et al., 1993). Thus, to increase energy expenditure and preclude hibernation, hamsters were cold-housed at 5 °C, a temperature that has been documented to increase thermogenic capacity, increase overall daily energy expenditure, and decrease body weight and adiposity, even in ad libitum-fed hamsters (Jefimow et al., 2004; Ruf and Graf, 2010; Schneider and Wade, 1990; Zhao, 2011). To prevent hibernation of cold-housed subjects, females were fed ad libitum and the duration of cold housing was limited to 7 days. Behavioral testing occurred during the last four days of this period.

*The preference apparatus*

A preference apparatus was designed to mimic important aspects of the hamsters' natural habitat and was used in experiments 1A and 2A to test both appetitive and consummatory aspects of sex and ingestive behavior. Syrian hamsters are solitary, live in deep burrows, are active above ground for short time periods, and travel long distances to their food source, filling their large cheek pouches with food, and transporting the food back to their burrows (Gattermann et al., 2008). At least one female has been observed mating near the

entrance to her burrow (Gattermann et al., 2008). Thus, the preference apparatus consisted of a home cage, with tunnels leading in one direction to a food source box, and in the other direction to a box that contained an adult male hamster (Schneider et al., 2007). Subject females lived in the home cage when they were not being trained or tested.

Preference tests occurred during the 90 min period that began at the onset of the dark phase of the photoperiod. The timing of the preference test was dictated by a combination of the observations made of Syrian hamsters living in the wild and in our laboratory. In their natural habitat, females spend about 90 min per day outside the burrow at dawn and dusk, and their time outside the burrow is thought to be limited due to predators or climate (Gattermann et al., 2008). In the laboratory, they are known to be nocturnal. We observe that when males are present, appetitive sex behaviors peak within the first 15 min and food hoarding peaks within 90 min of the onset of the dark phase of the photoperiod (Klingerman et al., 2010).

The home cage was made from opaque, Nalgene cages (31 × 19 × 18 cm) lined with fine ground wood shavings, equipped with a water bottle, standard laboratory chow (Harlan Rodent Chow #2016), and a

door at one end. When opened, the door led to a 134 cm-long tube that led from the home cage to two horizontal tubes 40–50 cm in length, connected in a T configuration.

One horizontal tube was connected to the food box, a disk-shaped, clear plastic chamber that contained a weighed amount of hoardable pellets but no water. Hoardable pellets were made from standard laboratory chow pellets cut into 2 cm pieces, a size that readily fits into Syrian hamster cheek pouches and allows the females to travel through the tubes with their cheek pouches full.

The second horizontal tube was connected to the male box, made of clear Plexiglass (27 × 20 × 15 cm), which contained an adult, sexually-experienced male Syrian hamster, no food or water, plus 50 mL of male bedding. This male bedding was collected prior to the start of the experiment from 6 or more different soiled home cages, mixed, and kept frozen. Just prior to the start of each preference test, 50 mL of this bedding mixture was sprinkled in the male box to standardize the pheromonal contributions of the male stimulus across all female subjects. Within the male box, the male hamster was restrained within a smaller wire box that permitted olfactory, gustatory, auditory, visual, and limited tactile interaction. The male stimulus hamster could neither leave the male box, nor could he fight or mate with the female.

#### *Acclimation, baseline, and training*

Twenty-seven estrous cycling female hamsters, between 113.8 and 180.4 g in body weight, were acclimated and trained in the preference apparatus at  $22 \pm 1$  °C. Female subjects were acclimated and trained in their own individual home cages attached to their own food or male boxes. First, female subjects were placed in the home cage with fine wood chip bedding, food, and water for at least 4 days (some were acclimated for up to 8 days) with the door closed and no access to the male or to the food boxes. This allowed them to establish their home territory, a process that involves choosing a corner for urination and a separate corner for sleeping and hoarding food. This acclimation reduces any tendency to carry bedding or food away from the home cage to the male or food boxes. Baseline measures of daily food intake and body weight were recorded.

Once acclimated to the home cages, hamsters received daily training sessions for four days in the preference apparatus. Training ensured that, at the start of behavioral testing, they would know which direction to search for food and which direction to search for the male. In this experiment, training occurred prior to cold exposure to minimize experimenters' time in the cold. Training sessions began within 1 h of the onset of the dark phase of the photoperiod and extended 1 to 2 h into the dark period. Training with the food box occurred on the post-ovulatory day and on follicular day 1 of the 4-day cycle. Females were allowed to discover the food boxes and to keep all of the food that they hoarded into their home cages. Training with the male box was on follicular day 2 and the periovulatory day. The females were allowed access to the male box for 2–5 min, where they were allowed to interact with an unrestrained adult, sexually-experienced male under close supervision by the experimenter. The female subject hamsters were allowed to receive anogenital sniffs, licks, and ectopic mounts but no intromissions or ejaculations from the male stimulus hamsters during training, but not during testing.

#### *Treatment*

The hamsters were divided into two groups that did not differ significantly in body weight prior to treatments. Half of the hamsters ( $n = 13$ ), the control group, remained in a room with an ambient temperature of  $22 \pm 1$  °C. Hamsters in the cold-housed experimental group ( $n = 14$ ) were placed in a refrigerated microenvironment maintained at  $5 \pm 2$  °C. Females were fed ad libitum throughout the experiment. Estrous cycles were not synchronized, and, on each day of testing, there was at least one animal representing each day of the estrous cycle.

#### *Behavioral testing*

Previous work shows that female Syrian hamsters begin to overeat 4 days after the start of cold housing indicating their response to increased energy demands for thermogenesis, and thus, on the 4th day of cold acclimation, daily preference testing began and continued for 4 days. All tests began within 1 h of the onset of the dark phase of the photoperiod. To start the test, the door to the vertical tube was opened and the female subjects were allowed access to both the food and male boxes for a total of 90 min. The female had the option of eating or hoarding food, visiting the male, or remaining in the home cage or the tubes. Within the male box, the stimulus male hamster was restrained in a wire mesh cage, and was not allowed to mate or fight with the female. On all days except the periovulatory day, the behaviors and locations were recorded by the experimenter every 5 s for 15 min. The recorded behaviors included, vaginal marking, flank marking, food hoarding, and eating.

Females display a different set of behaviors on the periovulatory day. They do not show vaginal scent marking, and they do show pre-lordosis and lordosis, behaviors absent on the other three days. Thus, on the periovulatory day, the experimenter recorded lordosis, a stationary, dorsoflexion of the spine with the tail positioned vertically, which allows male intromission. The experimenter also recorded the occurrence of pre-lordosis, in which the female stands stationary with the spine either straight or slightly ventroflexed with the tail in the horizontal position. After the first 10 min of the lordosis test, a separate 5-min test occurred in which the female was allowed to remain with the male while the experimenter physically stimulated the female's flanks, eliciting the lordosis reflex. The experimenter recorded the behavior of the female every 5 s for 5 min.

At the end of 15 min, the experimenter stopped recording, and the females continued to have access to both the food box and the male box for an additional 75 min. At the end of the final 75 min (90 min total), the female was placed in the home cage with no access to the food and male boxes. The weight of the food in the apparatus (the home cage and the food box) was measured and recorded. This gave a measure of 90-min food hoarding and food intake in the context of an available mating partner.

Four trained observers recorded behaviors. Two observers worked in the cold room while 2 worked in the warm room simultaneously.

After the last test, the females were euthanized on the next day. Female body weight and weight of the uterus and individual white adipose tissue (WAT) pads were measured to the nearest 0.001 g. WAT pads included the visceral (omental plus mesenteric), parametrial (gonadal) and subcutaneous (femoral) pads.

#### *Experiment 1B: Effects of ambient temperature on hormone concentrations over the estrous cycle*

A separate cohort of female hamsters was used to examine the effects of cold housing on plasma estradiol, progesterone, and leptin concentrations.

Baseline daily food intake was measured for four days by giving a weighed amount of food in the home cage and weighing the food remaining (minus pouched and spilled food) the next day. The hamsters were divided into 2 treatment groups that did not differ significantly in body weight or food intake ( $n = 20$  per group): Group 1, the control group, was housed at 22 °C, whereas Group 2, the experimental group, was housed at 5 °C. Treatments continued for 8 days. All hamsters were fed ad libitum, i.e., they had more than their daily food intake placed into the cage each day (9.54–10.29 g). Within each group of 20 hamsters there were 5 individuals that represented each day of the four-day estrous cycle. Thus, 5 females were euthanized on the first, 5 on the second, 5 on the third, and 5 on the fourth day of the estrous cycle. The start of cold or warm-housing was timed so that all animals were euthanized on the 8th day of their respective housing. At the end of the treatment period, animals were anesthetized with an overdose of sodium



pentobarbital (0.36 mL/100 g of body weight) at the onset of the dark phase of the light:dark cycle, the same time that behavior was tested in experiment 1A. Three milliliters of blood were collected by cardiac puncture and plasma was extracted. Plasma was analyzed for estradiol and progesterone by radioimmunoassay (RIA) (TKE21 and TKPG2, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Samples were run in duplicate for both estradiol and progesterone assays. The reportable range for the estradiol assay was 12.3–1700.0 pg/mL. For the progesterone assay, plasma was diluted to a 1:10 concentration in 0.1 M PBS with 1% BSA to allow values to lie within the reportable range of 0.07–22.0 ng/mL. The assays for estradiol and progesterone were conducted by the University of Virginia for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA, USA) used previously in Syrian hamsters (Klingerman et al., 2011b). Plasma was analyzed for leptin using the Multispecies RIA kit used previously in Syrian hamsters (Millipore (formerly Linco), St. Charles, MO, USA) (Schneider et al., 2000). Plasma samples were run in duplicate.

#### *Experiment 2A: Effects of access to running wheels on behavior over the estrous cycle*

This experiment was designed to determine whether estrous cycle fluctuations in appetitive behaviors are amplified when animals are provided with access to running wheels for voluntary locomotion. Two groups of hamsters were acclimated and housed at room temperature. One group was housed with access to running wheels (the experimental group) and one group without access to running wheels (the control group) for 10 days. All females were trained, and then tested in the preference apparatus (Fig. 1).

#### *Acclimation, baseline, and training*

Sixteen female hamsters 109.3–124.5 g in body weight were acclimated for 7 days to cages of the same size, at the same ambient temperature, with the same light–dark cycle as in experiment 1A. During the acclimation period, baseline daily food intake was determined for each hamster, i.e., a 4-day average daily food intake for each hamster was calculated. They were trained for 4 days in the preference apparatus as in experiment 1A. In this experiment, training occurred during 90-min sessions on each of four nights of the treatment period.

#### *Treatment*

The hamsters were divided into 2 groups that did not differ significantly in body weight. Females in the running-wheel group were individually housed in home cages equipped with a second door that led to an exercise wheel. The running wheels were available 24 h each day for the 10-day treatment period, except during the 90-min period when training and testing occurred (the last 6 days of treatment). All females in the running-wheel group were observed to run every night beginning several minutes before the onset of the dark phase of the light–dark cycle. To document that all females in the experimental group chose to run each night, those in the running-wheel group were video recorded for 15 min each night. Videos were taken within an hour of the onset of the dark period, before the start of the preference test. The sedentary group was housed in the same sized cage as the wheel-running group, but the cage had no extra door leading to a running wheel.

Preliminary observations showed that female hamsters immediately increase food intake to compensate for increases in energy expended on running. Thus, in the present experiment, to increase the likelihood that voluntary wheel running would pose an energetic challenge, hamsters in the running wheel group were not permitted to compensate for their wheel running by increasing their food intake. The wheel-running group was fed a daily ration of food that did not differ significantly from that of the control females housed without access to wheels.

#### *Behavioral testing*

Behavioral testing occurred in the preference apparatus as described in experiment 1A during the last 6 days that the wheel group had access to running wheels. Only the last four days of data were used in the analysis. Four trained observers recorded behaviors. Two observers worked with the wheel-housed females while 2 worked simultaneously with the females housed without access to running wheels.

After the last test, the females were euthanized on the next day. Uterine weight, body weight, and weight of the individual WAT pads were measured as described in experiment 1A.

#### *Experiment 2B: Effects of access to running wheels on hormone concentrations over the estrous cycle*

A separate cohort of female hamsters was used to examine the effects of running wheel access on plasma estradiol, progesterone, and leptin concentrations.

Baseline food intake was measured for 4 days. The hamsters were divided into 2 treatment groups that did not differ significantly in body weight or food intake ( $n = 20$  per group): Group 1 was housed at 22 °C with access to running wheels, whereas Group 2 was housed at 22 °C without running wheels. During the treatment period, food intake of the two groups did not differ significantly, as those without running wheels were fed ad libitum and those with running wheels were limited to that of the group housed without access to wheels.

This experiment was run simultaneously with experiment 1B, and the control group housed without running wheels at 22 °C and fed ad libitum is the same control group used for comparison in experiment 2B. Treatments continued for 8 days. Within each group of 20 hamsters there were 5 individuals that represented each day of the four-day estrous cycle. The start of the treatment period was timed so that all animals were euthanized on the 8th day of their respective housing.

At the end of the treatment period, animals were anesthetized with an overdose of sodium pentobarbital (0.36 mL/100 g of body weight) at the onset of the dark phase of the light:dark cycle, the same time that behavior was tested in experiment 2A. Three milliliters of blood were collected by cardiac puncture and plasma was extracted. Plasma was analyzed for estradiol, progesterone, and leptin by the assays described in experiment 1B.

#### *Statistical analyses*

Raw food hoarding scores were transformed to the natural log of the raw score + 1, so that the data would meet the assumption of homoskedasticity. Raw scores are shown in all figures for ease of presentation. Male preference was calculated as ((time spent with males minus the time spent with food) divided by the total time).

For the amount of food hoarded, the number of vaginal and flank marks, and male preference, results were analyzed using a two-way, repeated measures analysis of variance (ANOVA) with days of the estrous cycle as the repeated effect and treatment (cold vs. warm or wheel vs. no wheel) as the other effect. For the main effects, the effect sizes were calculated using Eta-squared,  $\eta^2$ . Post hoc comparisons were analyzed by Scheffe's F-test when main effects were significant. Differences were considered statistically significant if  $P$  was less than 0.05. The effect sizes for pair-wise comparisons were analyzed using Cohen's  $d$ .

One-way ANOVA was used to analyze plasma hormone concentrations and changes in body weight in experiments 1B and 2B, as blood samples for each day of the estrous cycle were obtained from separate groups of animals (blood was not sampled more than once for any animal).

Pearson's product moment scores ( $r^2$ ) are shown for the correlations between WAT pad weights and behaviors.

## Results

### Experiment 1A: Effects of ambient temperature on behavior over the estrous cycle

#### Ingestive behaviors at cold and warm ambient temperatures

The amount of food hoarded in 90 min ( $\ln(\text{raw score} + 1)$ ) changed significantly over the estrous cycle, with the lowest level of food hoarded on the periovulatory day (Fig. 2, top). Levels of 90-min food hoarding were higher in cold-housed compared to warm-housed females, and the effect of the estrous cycle on food hoarding was greater in cold-housed females (Fig. 2, top). In contrast, the warm housed females did not differ over the estrous cycle in levels of food hoarding (Fig. 2, top). This is confirmed by the repeated measures ANOVA, which showed a significant effect of estrous cycle day ( $F(3,75) = 28.73, P < 0.0001, \eta^2 = 0.18$ ), a significant stimulatory effect of ambient temperature ( $F(1,75) = 80.185, P < 0.0001, \eta^2 = 0.59$ ) and a significant interaction between estrous cycle day and ambient temperature ( $F(3,75) = 11.84, P < 0.0001, \eta^2 = 0.07$ ).

The average daily 90-min food hoarding on days 1–3 of the estrous cycle was calculated. The difference between periovulatory food hoarding (food hoarding on estrous cycle day 4) and average of food hoarding on days 1–3 was calculated for each female (mean  $\pm$  S.E.M.: warm =  $2.31 \pm 1.144$ , cold =  $85.28 \pm 9.8$ ). The mean of this peri-to-postovulatory difference was significantly greater in cold-housed compared to warm-housed females ( $P < 0.0001; d = 3.20$ ). Food hoarding in cold-housed females was significantly greater than that in warm-housed females on every day of the estrous cycle (Fig. 2, top,  $P < 0.001, 0.003, 0.005$  and  $0.042$  for days 1–4 of the estrous cycle, respectively;  $d = 4.08, 3.139, 1.8, 1.74$ ).

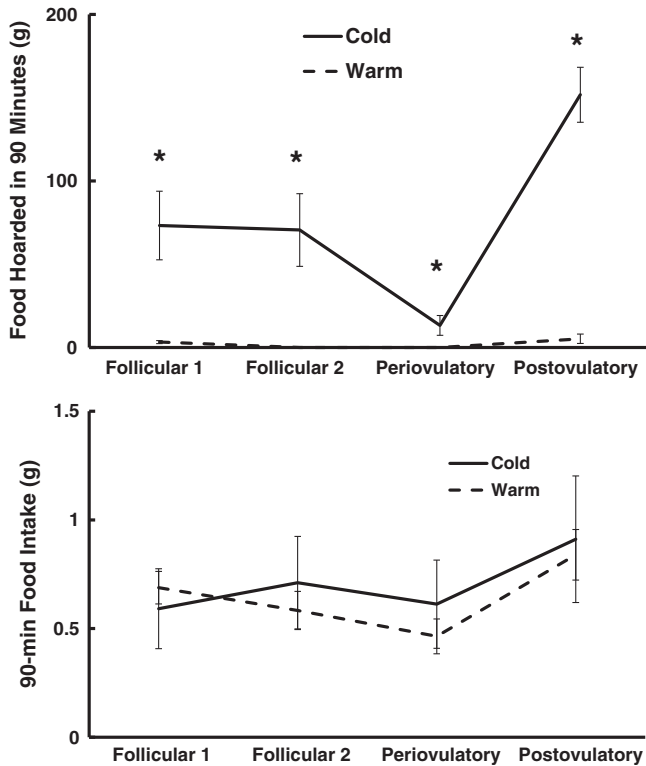


Fig. 2. Amount of food hoarded (top) and food intake (bottom) (mean  $\pm$  standard error of the mean, S.E.M.) for female Syrian hamsters tested during a 90-min period that spanned the onset of the dark phase of the photoperiod each day of the 4-day estrous cycle. Two groups were housed in one of two rooms maintained at either  $22 \pm 1^\circ\text{C}$  (warm) or at  $5 \pm 1^\circ\text{C}$  (cold) for 7 days before testing in experiment 1A. \* = significantly different from warm-housed females at  $P < 0.05$ .

Raw food hoarding scores were significantly negatively correlated with parametrial WAT pad weight ( $r^2 = 0.374, 0.338, 0.431$  and  $P < 0.01, 0.02, \text{ and } 0.01$  for the three nonestrous days of the cycle respectively) and subcutaneous WAT pad weight ( $r^2 = 0.326, P < 0.02$  on the postovulatory day), but not with the visceral WAT pad weight.

In contrast to 90-min food hoarding, 90-min food intake was not significantly affected by ambient temperature or the day of the estrous cycle (Fig. 2, bottom). The repeated measures ANOVA showed no significant effects of ambient temperature or day of the estrous cycle on 90-min food intake. Food intake was not significantly correlated with WAT pad weight except on the follicular day 2 when the raw food hoarding score was positively correlated with the weight of the parametrial WAT pad ( $r^2 = 0.293, P < 0.05$ ), i.e., the greater the WAT pad weight the higher the food intake.

Twenty four-hour food intake did not differ significantly between cold and warm-housed hamsters for the first 4 days of cold housing (mean  $\pm$  S.E.M. of cold vs. warm:  $10.69 \pm 1.22$  vs.  $10.17 \pm 1.04$ ), but during days 5 through 8, the cold-housed hamsters had significantly higher food intake compared to the warm-housed hamsters (mean  $\pm$  S.E.M. of cold vs. warm:  $15.00 \pm 0.49$  vs.  $9.5 \pm 0.28, P < 0.01; d = 5.2$ ).

#### Sex behaviors at cold and warm ambient temperature

Male preference changed significantly over the estrous cycle, and was lower in cold-housed than warm-housed females (Fig. 3, top). This is confirmed by the repeated measures ANOVA, which showed a significant effect of estrous cycle day ( $F(3,75) = 12.68, P < 0.0001; \eta^2 = 0.27$ ), a significant inhibitory effect of ambient temperature ( $F(1,75) = 12.18, P < 0.002; \eta^2 = 0.19$ ), and no significant interaction.

Male preference was significantly positively correlated with the weight of the parametrial WAT pad ( $r^2 = 0.116, P < 0.02$  for follicular day 2), the subcutaneous WAT pad ( $r^2 = 0.392, 0.486, P < 0.01, 0.01$  for follicular days 1 and 2), but not with the visceral WAT pad.

Levels of vaginal scent marking showed a similar pattern to male preference (Table 1). Repeated measures ANOVA showed a significant

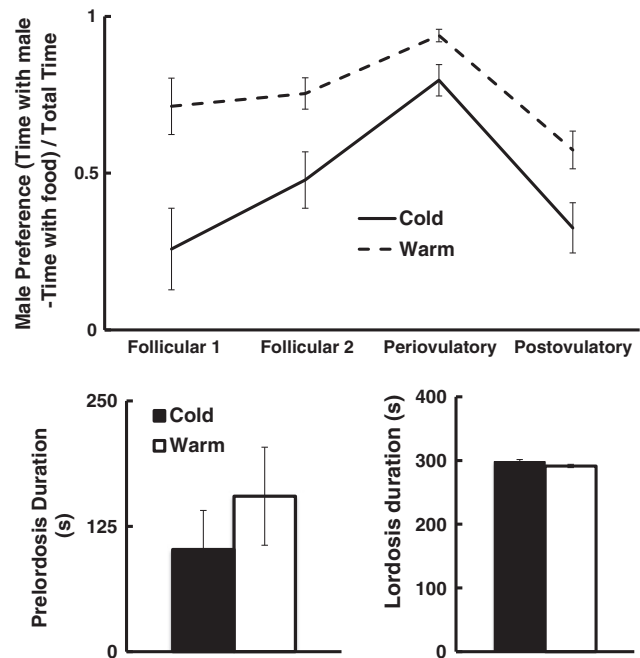


Fig. 3. Male preference (mean  $\pm$  S.E.M.), calculated as the ((time spent with males minus the time spent with food) divided by the total time) (top), and for lordosis duration and pre-lordosis duration (bottom) measured during a 15 min test each day of the 4-day estrous cycle. Female Syrian hamsters were housed in one of two rooms maintained at either  $22 \pm 1^\circ\text{C}$  (warm) or at  $5 \pm 1^\circ\text{C}$  (cold) for 7 days before testing in experiment 1A.

**Table 1**

Behaviors (means  $\pm$  S.E.M.) measured in experiment 1 in females housed either at cold ( $5 \pm 1^\circ\text{C}$ ) or warm ( $22 \pm 1^\circ\text{C}$ ) ambient temperatures and then tested in the preference apparatus on every day of the estrous cycle.

Behaviors measured in experiment 1	Estrous cycle day			
	Follicular day 1	Follicular day 2	Perioovulatory day	Postovulatory day
<i>Number of vaginal marks in 15 min (mean <math>\pm</math> S.E.M.)</i>				
Cold-housed	1.1 $\pm$ 0.6	11.9 $\pm$ 2.2	0.0 $\pm$ 0.0	0.3 $\pm$ 0.2
Warm-housed	3.4 $\pm$ 1.3	16.7 $\pm$ 3.4	0.0 $\pm$ 0.0	5.3 $\pm$ 2.1
<i>Number of flank marks in 15 min (means <math>\pm</math> S.E.M.)</i>				
Cold-housed	1.5 $\pm$ 0.7	1.3 $\pm$ 0.4	0.0 $\pm$ 0.0	0.4 $\pm$ 0.4
Warm-housed	5.5 $\pm$ 2.2	6.2 $\pm$ 2.2	0.2 $\pm$ 0.1	3.6 $\pm$ 1.6
<i>24-h food intake for the 1st 4 days (g)</i>				
Cold-housed	8.68 $\pm$ 0.66	10.59 $\pm$ 0.40	11.88 $\pm$ 0.29	11.59 $\pm$ 0.64
Warm-housed	8.51 $\pm$ 0.60	11.29 $\pm$ 0.44	10.71 $\pm$ 0.39	10.16 $\pm$ 0.37
<i>24-h food intake for the last 4 days (g)</i>				
Cold-housed	14.53 $\pm$ 0.52	15.88 $\pm$ 0.42	14.82 $\pm$ 0.84	14.77 $\pm$ 0.60
Warm-housed	10.12 $\pm$ 0.31	9.50 $\pm$ 0.31	9.21 $\pm$ 0.27	9.14 $\pm$ 0.41

inhibitory effect of ambient temperature ( $F(1, 75) = 4.7, P < 0.04; \eta^2 = 0.04$ ) and a significant effect of day of the estrous cycle ( $F(3, 75) = 33.6, P < 0.0001; \eta^2 = 0.54$ ) but no significant interaction. Peak levels of vaginal marking occurred on follicular day 2, the day before estrous.

Vaginal scent marking was significantly positively correlated with the parametrial WAT pad weight ( $r^2 = 0.359, 0.359, 0.433, P < 0.01$  for follicular days 1 and 2 and the postovulatory day, respectively), but not with the weight of the other WAT pads.

Levels of flank marking varied with ambient temperature and with estrous cycle day (Table 1), and the repeated measures ANOVA showed a significant inhibitory effect of ambient temperature ( $F(1, 75) = 4.58, P < 0.04; \eta^2 = 0.18$ ) and a significant effect of estrous cycle day ( $F(3, 75) = 7.56, P < 0.0002; \eta^2 = 0.18$ ) and a significant interaction ( $F(3, 75) = 2.81, P < 0.04; \eta^2 = 0.065$ ). The effects of the estrous cycle were most likely accounted for by the lack of flank marking on the day of estrous, since flank marking was similar on the other three days of the estrous cycle (Table 1).

The level of flank marking was positively correlated with the parametrial WAT pad weight ( $r^2 = 0.291, P < 0.03$ ) on follicular day 2 but not on any other day of the estrous cycle and not with any other WAT pad weight.

In contrast to purely appetitive sex behaviors discussed above, consummatory sex behavior, lordosis duration and pre-lordosis duration were not significantly affected by ambient temperature (Fig. 3, bottom). These consummatory behaviors were not significantly correlated with WAT pad weight.

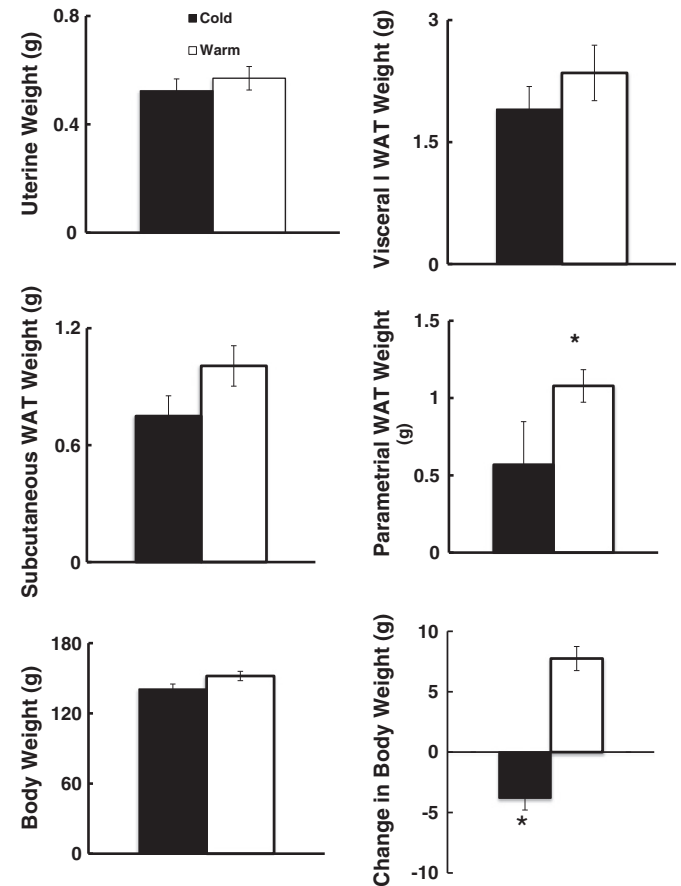
#### WAT pads and body weight at warm and cold temperatures

The two treatment groups did not differ significantly in body weight prior to treatment. Warm-housed females gained body weight, whereas cold-housed females lost body weight over the course of treatment. At the end of the experiment, cold-housed females had a significantly lower body weight ( $P < 0.006, d = 1.62$ ), and showed a significantly greater body weight loss ( $P < 0.0001, d = 1.24$ ) than warm-housed females. Cold-housed females also had significantly smaller parametrial WAT pad weights ( $P < 0.001; d = 2.23$ ) than cold-housed females, but the groups did not differ significantly in subcutaneous or visceral WAT pad weight. The groups did not differ in uterine weight, an index of circulating estradiol concentrations (Fig. 4).

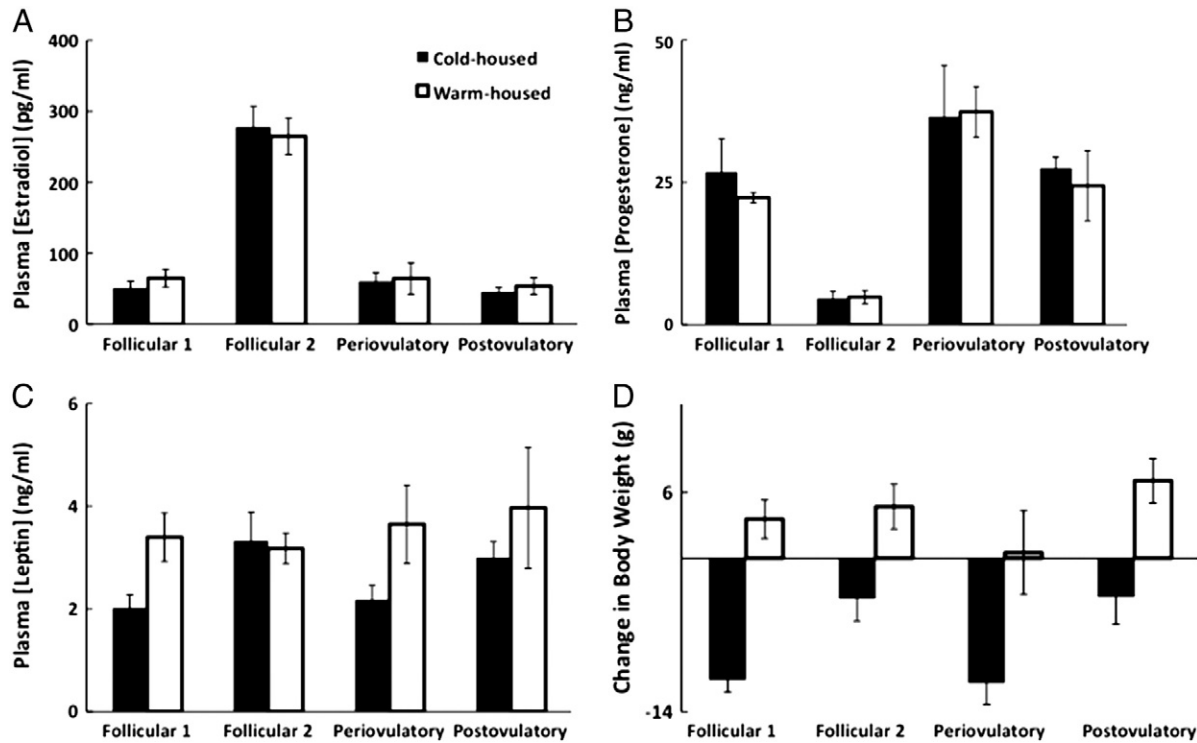
#### Experiment 1B: Effects of ambient temperature on plasma hormone concentrations

Plasma estradiol concentrations changed significantly over the estrous cycle, peaked on the day before estrous, and were not significantly affected by cold housing (Fig. 5A). This is confirmed by the two-way ANOVA, which showed a significant effect of estrous cycle

day ( $F(1, 30) = 64.08, P < 0.0001; \eta^2 = 0.86$ ), no significant effect of ambient temperature, and no significant interaction. In both treatment groups, plasma estradiol concentrations were highest during the night before estrous, and this level was significantly higher than on all other days ( $P < 0.0001; d = 5.71$  vs. day 1,  $d = 5.2$  vs. day 2,  $d = 4.37$  vs. day 4). All females in all groups showed this peak in estradiol concentrations except two cold-housed females that became anestrus during the last four days in the cold. All data from these two females were therefore excluded.



**Fig. 4.** Final body weight, uterine weight, and visceral, subcutaneous, and parametrial white adipose tissue (WAT) pad weights (mean  $\pm$  S.E.M.) from female hamsters that had been housed in one of two rooms maintained at either  $22 \pm 1^\circ\text{C}$  (warm) or at  $5 \pm 1^\circ\text{C}$  (cold) for 7 days before testing in experiment 1A. \* = significantly different from warm-housed females at  $P < 0.05$ .



**Fig. 5.** A. Plasma estradiol concentrations, B. plasma progesterone concentrations, C. plasma leptin concentrations, and D. change in body weight (means  $\pm$  S.E.M.) in female hamsters that had been housed in one of two rooms maintained at either  $22 \pm 1$  °C (warm) or at  $5 \pm 1$  °C (cold) for 8 days before testing in experiment 1B.

Plasma progesterone concentrations changed significantly over the estrous cycle, with a nadir at the time of peak plasma estradiol concentrations. Plasma progesterone concentrations peaked several hours after the time of ovulation, and were not significantly affected by cold housing (Fig. 5B). This is confirmed by the two-way ANOVA, which showed a significant effect of estrous cycle day ( $F(1,30) = 16.399$ ,  $P < 0.0001$ ;  $\eta^2 = 0.61$ ), but no significant effect of ambient temperature, and no significant interaction. In both temperature groups, plasma progesterone concentrations were lowest during the night before estrous, and this level was significantly lower than on all other days ( $P < 0.0001$ ;  $d = 3.04$  vs. day 1,  $d = 2.92$  vs. day 2,  $d = 3.35$  vs. day 4) and there were no significant differences among the other estrous cycle days.

Plasma leptin concentrations did not fluctuate significantly over the estrous cycle, but were significantly decreased by ambient temperature (Fig. 5C). This was confirmed by the ANOVA, which showed no significant effect of estrous cycle day, a significant inhibitory effect of ambient temperature ( $F(1,30) = 10.282$ ,  $P < 0.005$ ;  $\eta^2 = 0.22$ ), and no significant interaction.

There were no significant differences among the groups in body weight prior to treatment. Cold-housed hamsters weighed less than warm-housed hamsters at the end of the experiment, and the ANOVA showed a significant inhibitory effect of temperature ( $F(1,30) = 2.91$ ,  $P < 0.05$ ,  $\eta^2 = 0.22$ ). With regard to the change in body weight, the ANOVA showed no effect of estrous cycle day, a significant effect of ambient temperature ( $F(1,30) = 28.4$ ,  $P < 0.0001$ ;  $\eta^2 = 0.42$ ), and no significant interaction (Fig. 5D).

#### Experiment 2A: Effects of access to running wheels on behavior over the estrous cycle

##### Ingestive behavior in females with and without access to wheels

Food hoarding ( $\ln(\text{raw score} + 1)$ ) was not significantly affected by the presence of running wheels, but was significantly affected by the day of the estrous cycle. Repeated measures ANOVA showed no significant effect of being housed with a wheel and a significant

main effect of estrous cycle day on 90-min food hoarding (Fig. 6, top,  $F(3,42) = 2.75$ ,  $P < 0.05$ ;  $\eta^2 = 0.13$ ). The interaction was not significant.

Levels of food hoarding were not significantly correlated with the weights of any of the WAT pads.

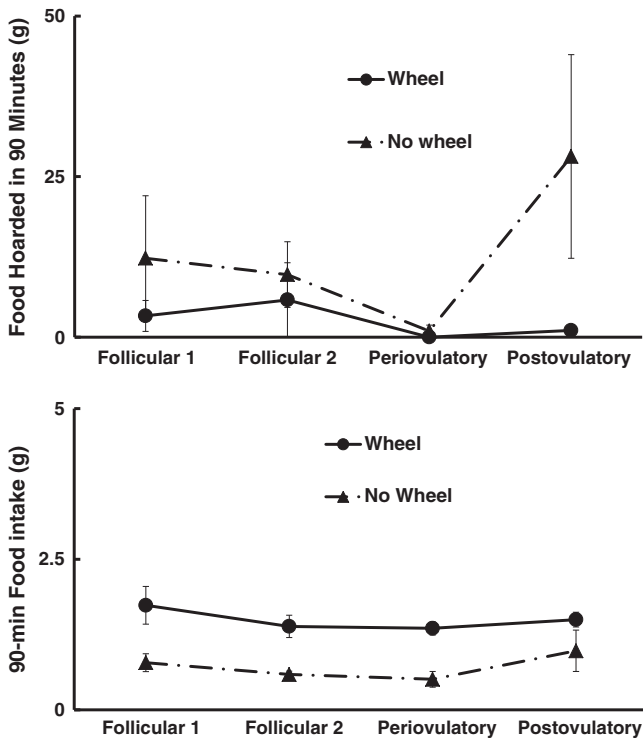
Ninety-minute food intake was significantly increased by the presence of running wheels, but was not significantly influenced by the day of the estrous cycle. Repeated measures ANOVA showed a significant stimulatory effect of access to wheels ( $F(1,42) = 16.31$ ,  $P < 0.001$ ;  $\eta^2 = 0.46$ ) and no significant effect of estrous cycle day (Fig. 6, bottom). Food intake during the testing period was significantly negatively correlated with the parametrial WAT pad weight on every day of the estrous cycle ( $r^2 = 0.237, 0.615, 0.647, 0.449$ ,  $P < 0.05, 0.003, 0.0002, 0.004$  respectively). Food intake was not significantly correlated with the weight of the other WAT pads.

##### Sex behavior in females with or without access to wheels

Male preference showed significant changes over the estrous cycle, and was significantly lower in females with access to wheels compared to females without (Fig. 7, top). Repeated measures ANOVA showed a significant effect of estrous cycle day ( $F(3,42) = 11.31$ ,  $P < 0.0001$ ;  $\eta^2 = 0.32$ ) and a significant inhibitory effect of access to running wheels on male preference (Fig. 7, top,  $F(3,42) = 26.86$ ,  $P < 0.0001$ ;  $\eta^2 = 0.24$ ) and no significant interaction. Male preference was significantly positively correlated with the weight of the parametrial WAT pad ( $r^2 = 0.299, 0.52$ ,  $P < 0.03, 0.002$  for the postovulatory day and follicular day 1 respectively), the weight of the subcutaneous WAT pad ( $r^2 = 0.392, 0.49$ ,  $P < 0.01, 0.002$  for the postovulatory day and follicular day 1 respectively), and the weight of the visceral WAT pad ( $r^2 = 0.384, 0.384$ ,  $P < 0.01, 0.01$  for the postovulatory day and follicular day 1 respectively).

The general pattern of vaginal scent marking was similar to that of male preference (Table 2). Levels of vaginal scent marking varied significantly over the estrous cycle as confirmed by the repeated measure ANOVA ( $F(3,42) = 8.06$ ,  $P < 0.0002$ ;  $\eta^2 = 0.35$ ), but there





**Fig. 6.** Amount of food hoarded (top) and food intake (bottom) (mean  $\pm$  S.E.M.) in hamsters tested during a 90 min period that spanned the onset of the dark phase of the photoperiod each day of the 4-day estrous cycle. Prior to testing the females were housed at 22 °C either with access to a running wheel or without access to a wheel for 6 days before testing (10 day total) in experiment 2A.

was no significant effect of access to running wheels on vaginal scent marking (Table 2).

Levels of vaginal scent marking were significantly positively correlated with the weight of the subcutaneous WAT pad ( $r^2 = 0.265$ ,  $P < 0.04$  on follicular day 1) and the weight of the visceral WAT pad ( $r^2 = 0.368$ ,  $P < 0.01$  on follicular day 1), but not on any other day of the estrous cycle and not with the weight of the parametrial WAT pad.

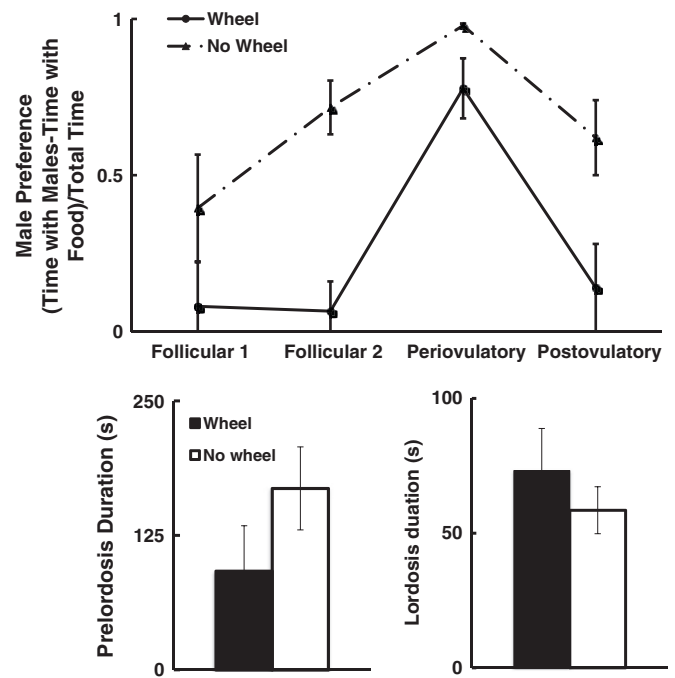
Levels of flank marking varied significantly over the estrous cycle, and females without access to wheels showed higher levels of flank marking than those with access to wheels (Table 2). There was a significant effect of day of the estrous cycle ( $F(3,42) = 4.28$ ,  $P < 0.01$ ;  $\eta^2 = 0.18$ ), and there was a significant inhibitory effect of access to running wheels ( $F(1,42) = 11.49$ ,  $P < 0.004$ ;  $\eta^2 = 0.12$ ) and no significant interaction.

Flank marking was significantly correlated with the weight of the subcutaneous WAT pad on follicular day 1 ( $r^2 = 0.492$ ,  $P < 0.003$ ), and was not significantly correlated with weight of the other WAT pads on any day of the estrous cycle.

Females with access to wheels did not differ from females without wheels in lordosis duration or in pre-lordosis duration (Fig. 7, bottom).

#### WAT pads and body weight in females housed with and without access to wheels

Females in the wheel and no wheel groups did not differ significantly in body weight at the start of the experiment, but at the end of the experiment, females with access to wheels had significantly lower body weights than females without access to wheels ( $F(1,14) = 29.97$ ,  $P < 0.0001$ ,  $\eta^2 = 0.68$ ). The females housed with running wheels lost significantly more body weight than those housed without access to wheels (Fig. 8,  $F(1,14) = 13.66$ ,  $P < 0.002$ ,  $\eta^2 = 0.49$ ). The visceral and parametrial (gonadal) WAT pad weights were significantly greater



**Fig. 7.** Male preference (mean and S.E.M.), calculated as the ((time spent with males minus the time spent with food) / the total time) from experiment 2. Preference was measured during a 15 min test each day of the 4-day estrous cycle. Female hamsters were housed either with or without access to a running wheel for 6 days before testing (10 day total) in experiment 2A.

in females housed without wheels compared to those housed with wheels (Fig. 8,  $F(1,14) = 17.59$ ,  $P < 0.001$ ;  $\eta^2 = 0.56$  for visceral and  $F(1,14) = 28.703$ ,  $P < 0.0001$ ;  $\eta^2 = 0.67$  for parametrial). Uterine and subcutaneous WAT pad weights were not significantly different between females with and without running wheels.

#### Voluntary wheel-running activity

Within the wheel-running group, one-way repeated measures ANOVA showed no significant change over the days of the estrous cycle in amount of time spent running in the wheels in a 15 minute observation period at the onset of the dark phase of the photoperiod (Table 2).

#### Experiment 2B: Effects of access to running wheels on plasma hormone concentrations

Plasma estradiol concentrations changed significantly over the estrous cycle, and were increased (not decreased) by housing the females with running wheels (Fig. 4). This is confirmed by the two-way ANOVA, which showed a significant effect of estrous cycle day ( $F(1,34) = 47.57$ ,  $P < 0.0001$ ;  $\eta^2 = 0.76$ ), a significant stimulatory effect of housing with running wheels ( $F(1,34) = 4.462$ ,  $P < 0.04$ ;  $\eta^2 = 0.023$ ), and no significant estrous cycle day by wheel interaction.

Plasma progesterone concentrations changed significantly over the estrous cycle, but were not significantly affected by housing with running wheels (Fig. 4). This is confirmed by the two-way ANOVA, which showed a significant effect of estrous cycle day ( $F(1,33) = 32.8$ ,  $P < 0.0001$ ;  $\eta^2 = 0.72$ ), but no significant effect of access to wheels, and no significant interaction.

Plasma leptin concentrations did not fluctuate significantly over the estrous cycle, but were decreased by housing with running wheels. This was confirmed by the ANOVA, which showed no significant effect of estrous cycle day, a significant inhibitory effect of housing with wheels ( $F(1,30) = 5.41$ ,  $P < 0.03$ ;  $\eta^2 = 0.14$ ), and no significant interaction.

**Table 2**  
Behaviors (means  $\pm$  S.E.M.) measured in experiment 2 in females housed either with or without running wheels and then tested in the preference apparatus on every day of the estrous cycle. In the wheel-housed group, food was limited to that of the control group so that there were no significant differences in 24-hour food intake.

Behaviors measured in experiment 2	Estrous cycle day			
	Follicular day 1	Follicular day 2	Perioviulatory day	Postovulatory day
<i>Number of vaginal marks in 15 min (mean <math>\pm</math> S.E.M.)</i>				
Wheel	3.6 $\pm$ 1.8	6.6 $\pm$ 2.3	0.0 $\pm$ 0.0	0.4 $\pm$ 0.3
No-wheel	6.9 $\pm$ 2.1	5.6 $\pm$ 1.8	0.0 $\pm$ 0.0	2.2 $\pm$ 1.1
<i>Number of flank marks in 15 min (mean <math>\pm</math> S.E.M.)</i>				
Wheel	1.0 $\pm$ 0.5	1.0 $\pm$ 0.6	0.0 $\pm$ 0.0	0.2 $\pm$ 0.1
No-wheel	7.6 $\pm$ 3.6	6.0 $\pm$ 1.5	0.0 $\pm$ 0.0	0.9 $\pm$ 0.4
<i>Number of wheel revolutions in 15 min</i>				
Wheel-running	6.25 $\pm$ 0.45	5.83 $\pm$ 0.33	6.64 $\pm$ 0.32	5.85 $\pm$ 0.26

There were no significant differences among the groups in body weight at the start of treatments. Change in body weight over treatment was calculated as final body weight minus starting body weight, and the ANOVA showed no effect of estrous cycle day, a significant inhibitory effect of housing with running wheels ( $F(1,34) = 27.98, P < 0.0001; \eta^2 = 0.41$ ), and no significant interaction.

## Discussion

The main finding was that housing at cold ambient temperatures with ad libitum food intake unmasked estrous cycle effects on sex and ingestive behavior that were obscured in typical laboratory conditions characterized by isolation in a small cage, warm ambient temperatures, and ad libitum access to food (Figs. 2 and 3). The effect of cold housing was significant for the appetitive behaviors, food hoarding, male preference, and vaginal scent marking, but not for consummatory behaviors, food intake and lordosis duration. In females housed at 5 °C, high levels of sexual motivation were restricted to the perioviulatory day, but on the three nonestrous days, these females showed high levels of food hoarding, but not food intake during the 90 min testing period. This was in contrast to females housed at 22 °C, which showed high levels of sexual motivation and low levels of food hoarding on every day of the cycle.

These effects of cold housing are likely to be mediated by the increased energy expended on thermogenesis. Indirect evidence for increased energy expenditure in the cold-housed females is provided by the fact that they lost body weight and showed significantly lower WAT pad weights and plasma leptin concentrations than warm-housed females (Figs. 4 and 5). More direct evidence is provided by previous work, which showed that cold-housing in this species results in significant increases in thermogenic capacity, increases in overall daily energy expenditure, and decreases in body weight and adiposity (Jefimow et al., 2004; Ruf and Graf, 2010; Schneider and Wade, 1990; Zhao, 2011). Furthermore, the effects of housing at 5 °C with ad libitum food intake were remarkably similar to effects of housing at 22 °C with 25% food restriction (Klingerman et al., 2010).

Sexual motivation was also decreased by housing the females with running wheels at the warm ambient temperature (22°C) (Fig. 7), but there were important differences between effects of cold housing and access to running wheels (Table 3). Whereas cold-housed females showed significantly elevated levels of food hoarding on the nonestrous days, those housed with access to running wheels showed low levels of food hoarding that were not significantly different from those shown by females housed without access to running wheels. By contrast to cold-housed females, those housed in the warm with running wheels increased food intake during the 90 min test, and this occurred on every day of the estrous cycle (Table 3). The females were not forced to run in their wheels, and when their food intake was limited to that of the no-wheel controls, they could have chosen to conserve energy by limiting their running rather than by decreasing their time spent with males or by increasing their food intake during the 90-min testing period. By sharp contrast to cold-housed females, wheel-housed females showed significant increases in food intake, but did not increase food hoarding. Furthermore, the level of food intake, but not of food hoarding was significantly negatively correlated with the weight of the WAT pads. Together, these results suggest that when given the opportunity, females will voluntarily expend energy on locomotion, mobilize metabolic fuels from the lipid stores in WAT, and limit their appetitive sex behaviors to the perioviulatory period.

The effects of housing with access to running wheels on behavior is likely to be due to the increased energy expended on locomotion. Indirect evidence that the wheel running females experienced increased energy expenditure is provided by the fact that they lost an amount of body weight similar to that of the cold-housed females (Figs. 8 and 9) and showed significantly lower WAT pad weights and plasma leptin concentrations than warm-housed females without access to wheels (Fig. 9). The introduction of running wheels to Syrian hamsters is known from previous studies to increase locomotion throughout the dark phase of the photoperiod, increase overall energy expenditure, increase food intake, and decrease body weight and adiposity (Coutinho et al., 2006; Davis et al., 1987; Refinetti and Menaker, 1997; Richards, 1966). Furthermore, in a different hamsters species (*P. sungorus*),

**Table 3**  
Results from the repeated measures ANOVA used to analyze effects of treatments, estrous cycle day, and the interaction of treatment  $\times$  estrous cycle day for behaviors measured in experiments 1 and 2. In experiment 1, the main effect of treatment refers to housing at either cold or warm ambient temperatures. In experiment 2, the main effect of treatment refers to housing with or without access to running wheels. \* = significant effect at  $P < 0.05$  and – = the effect was not significant.

Behaviors/effects	Experiment 1			Experiment 2		
	Cold vs. warm	Estrous cycle day	Interaction	Wheel vs. no wheel	Estrous cycle day	Interaction
Food hoarding	*	*	*	–	*	–
Male preference	*	*	–	*	*	–
Vaginal marking	*	–	–	*	–	–
Food intake	–	–	–	*	–	–
Lordosis duration	–	Only on estrous	n/a	–	Only on estrous	n/a

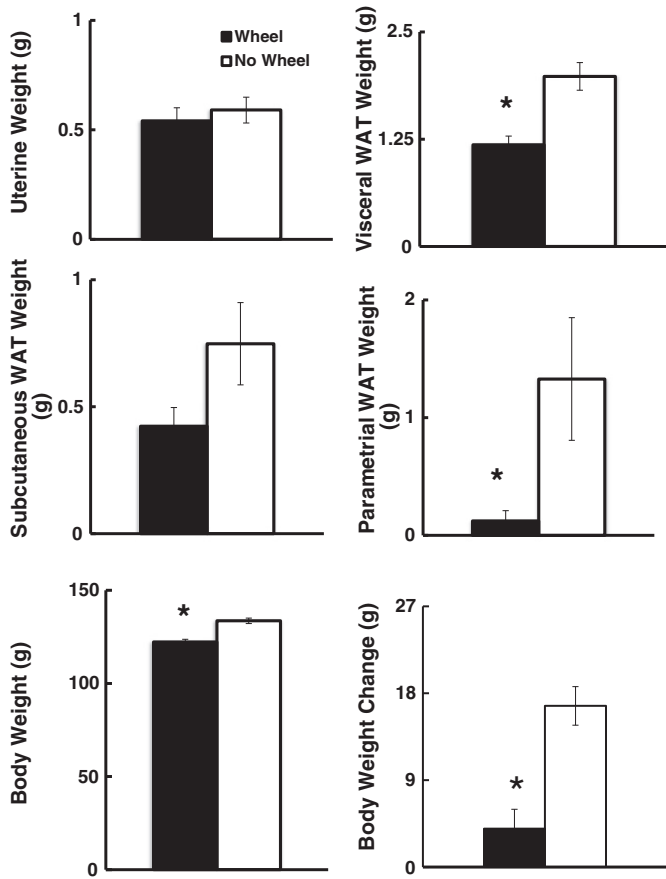


Fig. 8. Final body weight, uterine weight, and visceral, subcutaneous, and parametrial white adipose tissue pad weights (mean and S.E.M.). Female hamsters were housed either with or without access to a running wheel for 6 days before testing (10 days total) in experiment 2A. \* = significantly different from warm-housed females at  $P < 0.05$ .

voluntary wheel running attenuates many aspects of reproduction (Petri et al., 2010).

Cold-induced and wheel running-induced changes in behavior were not linked to decreases in estradiol and progesterone concentrations induced by those energetic challenges. There were no significant differences between cold and warm housed females, or between wheel-housed and no-wheel hamsters in uterine weight (Figs. 5 and 9), a measure that is significantly correlated with circulating concentrations of estradiol (Schatz et al., 1983). In experiments 1B and 2B, there were no significant effects of the energetic challenges on plasma estradiol or progesterone concentrations (Figs. 5 and 9). In cold-housed, wheel-housed and warm-housed females without wheels, plasma estradiol concentrations were highest and progesterone concentrations were lowest on the evening of follicular day 2 of the cycle, consistent with the literature on this species (Shaikh, 1972). Cold ambient temperature, housing with running wheels, and 25% food restriction (data not shown) had no significant effect on these parameters. These data are consistent with previous experiments, which show that exogenous ovarian steroid treatment significantly increases male preference in food-restricted females, but has little or no effect in females fed ad libitum (Klingerman et al., 2010). Thus, even when steroids are held constant by exogenous manipulation, there are differences in behavior depending on the energetic status of the female at the time of steroid treatment. In summary, the effects of energetic challenges are not attributable to changes in circulating levels of ovarian steroids, but to differences in the response or sensitivity to those steroids.

Effects of cold housing and access to wheels on appetitive sex behavior might be related to significantly reduced plasma leptin concentrations in the energetically challenged groups (Figs. 5 and 9). While these correlations cannot be taken as evidence for causation, other studies show that systemic leptin treatment can attenuate food deprivation-induced food hoarding in the absence of opposite-sex conspecifics (Buckley and Schneider, 2003), and reverse food deprivation-induced deficits in appetitive sex behavior in Syrian hamsters tested in the absence of food (Schneider et al., 2007). Together, these results are

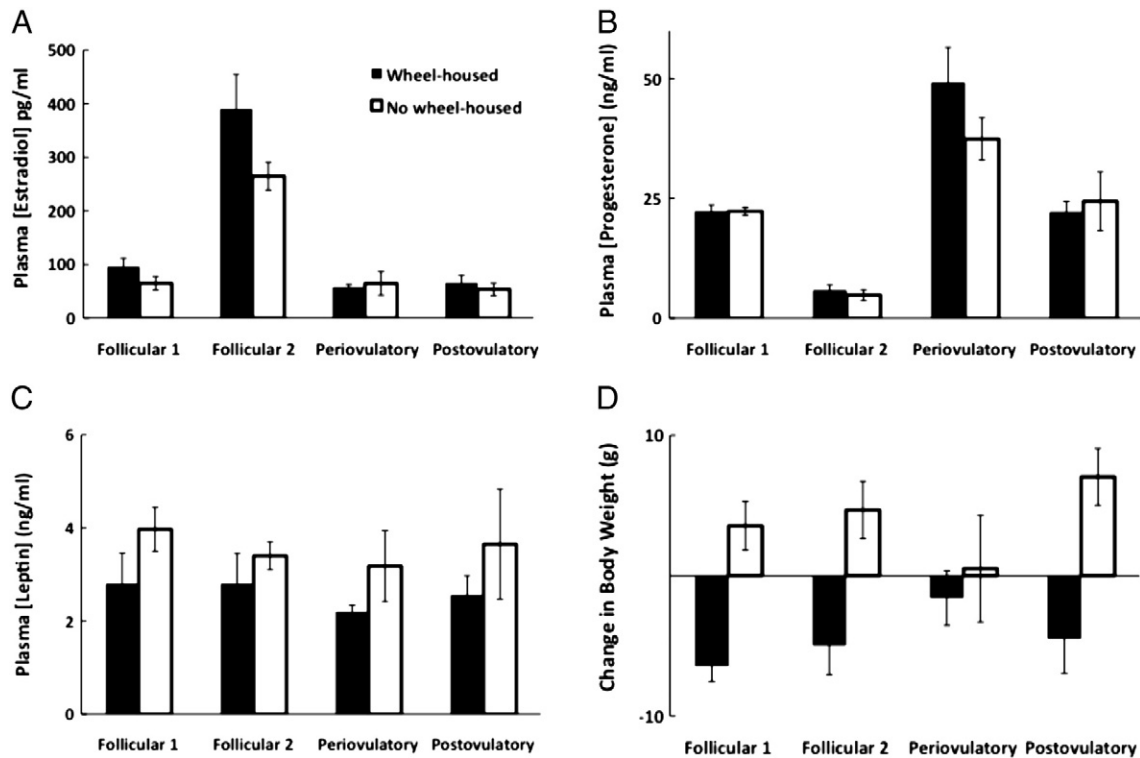


Fig. 9. A. Plasma estradiol concentrations, B. plasma progesterone concentrations, C. plasma leptin concentrations, and D. change in body weight (means  $\pm$  S.E.M.) in female hamsters that had been housed in one of two rooms, one with and one without access to running wheels for 8 days before testing in experiment 1B.

consistent with the idea that ovarian hormones that fluctuate over the estrous cycle interact with the overall availability of metabolic fuels (a function of food intake and body fat storage minus energy expenditure) and/or plasma leptin concentrations.

In food-restricted females in a previous study (Klingerman et al., 2010), and in cold housed females in this study, low plasma leptin concentrations, normal ovarian steroid concentrations, and a mild energetic challenge led to increased food hoarding. This same combination of factors led to increased food intake and low food hoarding in the hamsters housed with running wheels. Thus, there is something peculiar to exercise in general or wheel-running in particular that increases food intake and decreases food hoarding. Housing with running wheels is known to produce a unique array of hormone and neuropeptide changes (Novak et al., 2012), and these other hormonal adjustments might also account for fluctuations in food intake vs. food hoarding in the wheel-housed group.

It is unlikely that ad libitum-feeding of the wheel-housed group would have resulted in patterns of food hoarding similar to the cold-housed or food-restricted females, because the energy expended on wheel running would have been balanced by their increased intake, resulting in no net energy deficit, and thus, they would be expected to behave more like the no-wheel controls than like the energetically challenged groups.

All of the above results, together with other published work, suggest that food hoarding is important in relation to adaptation to cold and in preparation for sedentary periods that might include bouts of heterothermy, e.g., hibernation or torpor. Other heterothermic hoarding species also show cold-induced and food deprivation-induced food hoarding that is inhibited by wheel running. For example, in Siberian hamsters that are housed with strict foraging requirements, i.e., they are required to run many revolutions in running wheels in order to receive a fixed amount of food, food hoarding is increased by food deprivation, but the effect is attenuated in hamsters with the highest wheel-running requirement (Day and Bartness, 2001). Differences in the neuroendocrine mechanisms that underlie the differences in food hoarding response to ambient temperature vs. wheel running are under investigation.

Of the three WAT pads that were examined, the gonadal (parametrial) WAT pad was the most affected by cold housing and access to wheels, being the only WAT pad in the cold-housed females that was significantly lower than that of the warm-housed females (Fig. 4), and being almost completely depleted in the wheel-running females (Fig. 8). Similarly, parametrial WAT pads are the most affected by increased wheel-running in female Siberian hamsters required to forage for their food (Day and Bartness, 2001). WAT pad depletion that is specific to the gonadal pad might have important effects on gonadal function, HPG function, and/or behavior, but in this case, their depletion did not lead to significant decreases in plasma concentrations of estradiol or progesterone. Similarly, complete removal of the epididymal WAT pad in male Syrian hamsters inhibited spermatogenesis and follicle stimulating hormone (FSH) secretion, but not luteinizing hormone or testosterone secretion (Chu et al., 2010). The effects on spermatogenesis and gonadotropins do not occur with removal of any other WAT depot, suggesting that the presence of at least some gonadal WAT is permissive for FSH and spermatogenesis. It is not known whether parametrial WAT pad size influences gonadotropins or behavior in female Syrian hamsters, and the weight of this pad was associated with changes in behaviors with no changes in circulating steroid hormones. In any case, preferential loss of fat from the gonadal WAT pads during energetic challenges is consistent with the notion that the energy balancing system modulates reproductive success in environments where energy fluctuates. Cold-housing resulted in a significant loss of lipids in the parametrial WAT pad that shares a blood supply with the uterine horns, but there was no significant weight loss in the other WAT pads (Fig. 4). The sparing of the visceral WAT pad during cold acclimation (Fig. 4), but not during wheel running in the warm environment (Fig. 8), might be crucial for protecting the internal organs from

cold ambient temperature. The gonadal WAT pad might be less important for survival in the cold, and thus the cold-housed female might increase her chances of survival by preferentially drawing energy from these expendable stores while simultaneously decreasing sexual motivation.

In cold-housed females, the levels of food hoarding, but not food intake were significantly correlated with the weight of the WAT pads, particularly the weight of the parametrial WAT pad. In warm-housed females the opposite was true; the level of food intake, not food hoarding was correlated with the weight of the WAT pads. Running in wheels and living in the cold seems to affect the same WAT pad, and yet, something about running in wheels switched the strategic response from increases in appetitive behavior (food hoarding) to increases in consummatory behavior (eating). It is possible that running in wheels provided a rewarding stimulus that satisfied the need to engage in appetitive ingestive behavior (food hoarding). It would be interesting to examine whether there are functional connections among gonadal WAT pads, gonadotropins, mesolimbic dopamine, and appetitive and consummatory sex and ingestive behavior.

These results lead to additional testable hypotheses about the mechanisms by which estradiol orchestrates behavioral choice. For example, we hypothesize that during the early follicular phase of the estrous cycle when circulating estradiol levels are low, sexual motivation is tonically inhibited by one or a number of putative “orexigenic” peptides, perhaps ghrelin, GnIH, NPY, agouti-related protein (AgRP), endocannabinoids, or some combination of these. Furthermore, we hypothesize that periovulatory levels of estradiol disinhibit the effects of orexigenic hormones and neuropeptides in order to couple reproductive motivation with fertility and inhibit food hoarding. These hypotheses lead to testable predictions about the effects of estradiol on neural activation in identified neurons. Consistent with this idea, changes in cellular activation in hypothalamic GnIH cells are more closely associated with appetitive than consummatory sex and ingestive behaviors (Klingerman et al., 2011c) and treatment with GnIH inhibits appetitive sex behaviors (Piekarski et al., 2012) and increases food hoarding (Benton and Schneider, unpublished observations). It will be interesting to determine the effects of cold vs. exercise on cellular activation in GnIH-containing cells (and other peptides) to see whether they are more closely associated with changes in food hoarding or food intake. Further experiments will determine whether food hoarding patterns and patterns of activation of GnIH cells are flattened in cold-housed, wheel-housed hamsters, and food-restricted animals that are treated with leptin or exaggerated in ad libitum-fed females treated with ghrelin or metabolic inhibitors.

As suggested previously (Klingerman et al., 2010, 2011a,c), steroid effects on behavior are illuminated by experimental designs that include the energetic conditions and behavioral choices found in the natural habitat of the species under study, presumably because those conditions mimic the selection pressures that molded those behaviors during evolution. The present experiments confirm that it is important to quantify behavioral motivation, reflected in appetitive behaviors such as male preference and food hoarding, in addition to traditional measures of behavioral performance such as lordosis and food intake (Ball and Balthazart, 2008; Balthazart et al., 1995; Bartness et al., 2011; Keen-Rhinehart et al., 2013; Schneider et al., 2013). Furthermore, the effects of hormones on motivation are unmasked by mild energetic challenges. This suggests that ad libitum feeding should be seen not as a control condition, but rather, as one of many artificial, experimental manipulations. Would the effects of ovarian steroids on sexual libido be more widely recognized in societies where women engage in long hours of physical labor and expend a large portion of their energy budget to obtain and store food?

The present results have clinical relevance with regard to normal and disordered eating. For example, in women from modern, westernized societies, the menstrual cycle fluctuations in food intake



are subtle and difficult to replicate (Fessler, 2003), yet, in populations of women who tend to limit their food intake, binge eating is significantly increased during the phases of the menstrual cycle when estradiol is low and progesterone is high (Edler et al., 2007; Klump et al., 2008). Not all binge eaters limit their food intake, but the fact that low energy balance can exaggerate the effects of low estradiol on appetitive ingestive behavior in women is consistent with the present results. In summary, physiological mechanisms that were critical for survival and reproductive success during the evolution of species living in energetically labile habitats might be expected to share a common feature: interactive effects of ovarian steroids and energy balance on motivation (Schneider et al., 2013).

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